Keratinocyte-Derived Granulocyte–Macrophage Colony Stimulating Factor Accelerates Wound Healing: Stimulation of Keratinocyte Proliferation, Granulation Tissue Formation, and Vascularization

Amrit Mann, Kai Breuhahn,[1] Peter Schirmacher,* and Manfred Blessing

I. Medical Department, Johannes Gutenberg University, Mainz, Germany; *Institute of Pathology, University of Cologne, Cologne, Germany

Chronic, nonhealing wounds represent a major clinical challenge to practically all disciplines in modern medicine including dermatology, oncology, surgery, and hematology. In skin wounds, granulocyte–macrophage colony stimulating factor (GM-CSF) is secreted by keratinocytes shortly after injury and mediates epidermal cell proliferation in an autocrine manner. Many other cells involved in wound healing including macrophages, lymphocytes, fibroblasts, endothelial cells, and dendritic cells synthesize GM-CSF and/or are targets of this cytokine. Therefore, GM-CSF is a pleiotropic cytokine evoking complex processes during wound repair. Despite this complexity and the scarcity of mechanistic understanding GM-CSF has been employed in trials of clinical treatment of skin wounds with some success. In this study, we evaluated a transgenic mouse model in order to analyze the effects of an excess of keratinocyte-derived GM-CSF on excisional wound healing in the skin. Transgenic mice constitutively overexpressing GM-CSF in the basal layer of the epidermis displayed accelerated reepithelialization of full-thickness skin wounds. In the early stages of wound repair, transgenic mice exhibited significantly higher numbers of proliferating keratinocytes at the wound edges and increased formation of granulation tissue with enhanced neovascularization. As a potential mechanism of these beneficial changes, we identified the differential temporal regulation of cytokines such as transforming growth factor-β, a known angiogenetic factor, interferon-γ, a proinflammatory cytokine, and interleukin 6, an essential factor for reepithelialization, in transgenic mice *versus* controls. We propose that the beneficial effects observed in GM-CSF transgenics are due not only to direct GM-CSF action but in addition to indirect processes *via* the induction of secondary cytokines.

Key words: GM-CSF/skin/transgenic mice/wound healing.

J Invest Dermatol 117:1382–1390, 2001

Disruption of the integrity of the epidermis by wounding evokes a response involving both biochemical and cellular events designed to restore a functional epithelium over the wounded site (Clark, 1985). The initiation of wound healing involves sequential completion of coagulation, inflammation, and removal of damaged matrix components, followed by cellular proliferation and migration, angiogenesis, matrix synthesis and deposition, reepithelialization, and remodeling (Martin, 1997; Baker and Leaper, 2000). Thus, wound healing involves interactions between different cell types and matrix components as well as communication between different cell types themselves. The latter is mediated to a great extent by cytokines, growth factors, and their respective receptors. The impact of increased or decreased activity of some of these signaling molecules or signal transduction components on wound healing has been evaluated *in vivo* in transgenic or knockout animals. Examples include transforming growth factor α (TGF-α), TGF-β superfamily members, fibroblast growth factor family members, interleukins (ILs), chemokines, and the respective receptors (Grose and Sabine, 2001).

Very few of these proteins, e.g., platelet-derived growth factor (Rees *et al*, 1999), have ever been used in clinical settings. In contrast, granulocyte–macrophage colony stimulating factor (GM-CSF) has been shown to exert beneficial effects on wound healing in patients suffering from poorly healing wounds and chronic skin ulcers of diverse etiology, e.g., hydroxyurea-related leg ulcers (Stagno *et al*, 1999), venous leg ulcers (Da Costa *et al*, 1999), hemoglobinopathy-related ulcers (Voskaridou *et al*, 1999), and wounds resulting from amputation (Gaches *et al*, 1998). Furthermore, intradermal administration of GM-CSF to leprosy patients with skin lesions leads to enhanced wound healing and increased numbers and layers of keratinocytes (Kaplan *et al*, 1992; Braunstein *et al*, 1994). There is one study, however, where no beneficial effects of GM-CSF application were identified in normally healing wounds (Ure *et al*, 1998). The beneficial effects of GM-CSF have been associated with increased keratinocyte proliferation and increased breaking strength of wounds.

Upon epidermal activation such as injury or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treatment, GM-CSF mRNA

Manuscript received June 27, 2001; revised August 30, 2001; accepted for publication September 10, 2001.

Reprint requests to: Dr. Manfred Blessing, I. Medical Department, Johannes Gutenberg-University, D-55131 Mainz, Germany. Email: Blessing@mail.uni-mainz.de

[1]Present address: Institute of Pathology, University of Cologne, D-50931 Cologne, Germany.

accumulates in keratinocytes within a few hours (Robertson *et al*, 1994; Vasunia *et al*, 1994). GM-CSF is therefore an early response gene and may evoke a series of events eventually resulting in wound closure and tissue remodeling. GM-CSF is a potent mitogen for keratinocytes at nanogram per milliliter concentrations (Kawada *et al*, 1997) and it directly stimulates the migration and proliferation of human endothelial cells and the growth of human keratinocytes *in vitro* (Hancock *et al*, 1988; Bussolino *et al*, 1989). In addition, it is hypothesized that GM-CSF influences proliferation, maturation, and recruitment of cells like keratinocytes, fibroblasts, endothelial cells, monocytes, macrophages, and dendritic cells, at least in part by modulating the release of cytokines such as IL-1, IL-6, tumor necrosis factor α (TNF-α), TGF-β, interferon-γ (IFN-γ), and M-CSF, which in turn affect the healing process (Stagno *et al*, 1999).

There are animal models allowing the evaluation of exogenously applied GM-CSF or the transient, cutaneous expression of GM-CSF from an adenoviral vector (Jyung *et al*, 1994a; Xing *et al*, 1997a). For example, enhanced wound healing in a surgical incision model and steroid-treated monocyte-depleted animals by locally applied GM-CSF has been reported (Jyung *et al*, 1994a). There is no animal model, however, to evaluate (i) the consequences of long-term cutaneous overexpression of GM-CSF, (ii) the impact of excess keratinocyte-derived GM-CSF on wound healing, and (iii) the mechanisms by which this cytokine alters wound healing processes. The latter point is of exceptional interest, as GM-CSF is known both to be produced by and to target many different cell types involved in tissue repair including keratinocytes, fibroblasts, macrophages, and dendritic and endothelial cells (Kaplan *et al*, 1992; Braunstein *et al*, 1994; Jyung *et al*, 1994b; Bratton *et al*, 1995; Caux *et al*, 1996).

In this study, we used previously established transgenic mice epidermally overexpressing GM-CSF in cutaneous wound healing studies in order to address the above-mentioned questions (Breuhahn *et al*, 2000). In these transgenic mice, the wound healing process is accelerated as demonstrated by the extent of reepithelialization and scab rejection. At early stages of wound repair, mitotic indices in these mice were significantly higher than in wild-type mice. In addition, significantly increased neovascularization of wounds was observed in GM-CSF-overexpressing mice, which may be caused by the induction of TGF-β1 mRNA in skin wounds of these transgenics. Besides GM-CSF and TGF-β, IL-6, which is known to be essential for wound healing (Sano *et al*, 1999;

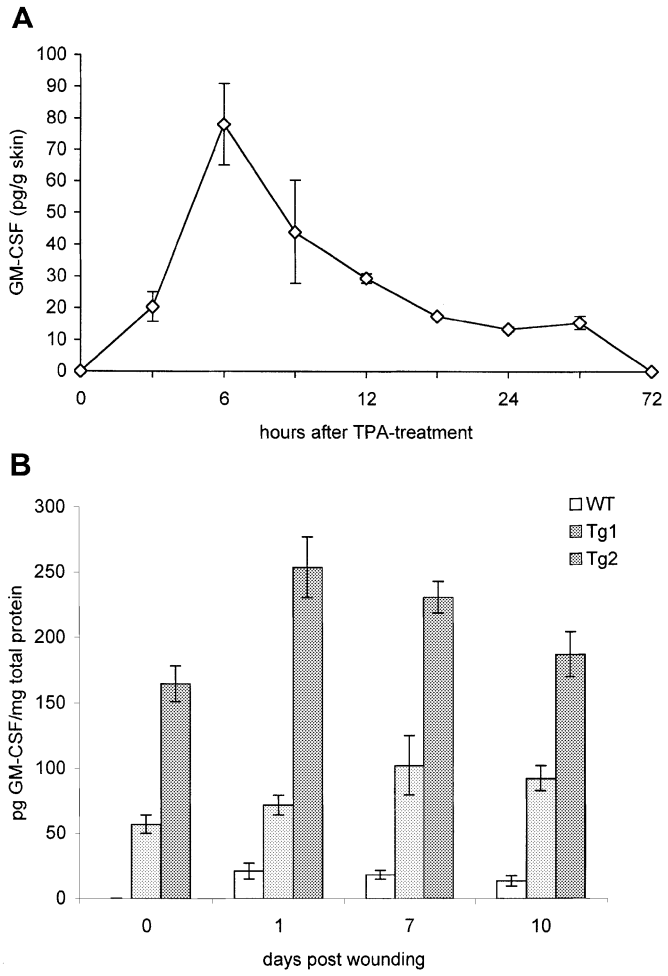


Figure 1. GM-CSF is induced in skin following TPA treatment or wounding. (*A*) Skin samples were collected at different time points after a single application of 2.5 μg TPA ($n = 3$ animals per experimental time point). GM-CSF protein content in skin extracts of wild-type mice was measured by ELISA. Strongest induction can be observed as soon as 6 h after the treatment. (*B*) GM-CSF protein content in skin extracts after wounding. Transgenic mice of lines Tg1 and Tg2 as well as wild-type littermates were wounded as described in *Materials and Methods*. Four specimens were pooled for each experimental time point for analysis. Skin extracts were prepared and GM-CSF levels were determined by ELISA. High levels of GM-CSF during the repair process can be observed in both transgenic lines Tg1 and Tg2, whereas the control animals showed much lower GM-CSF tissue levels.

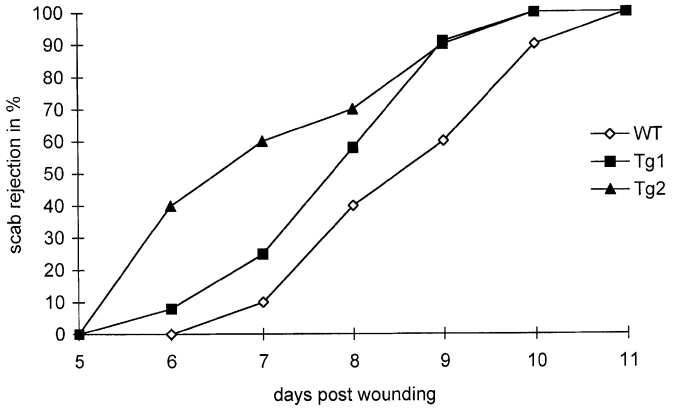
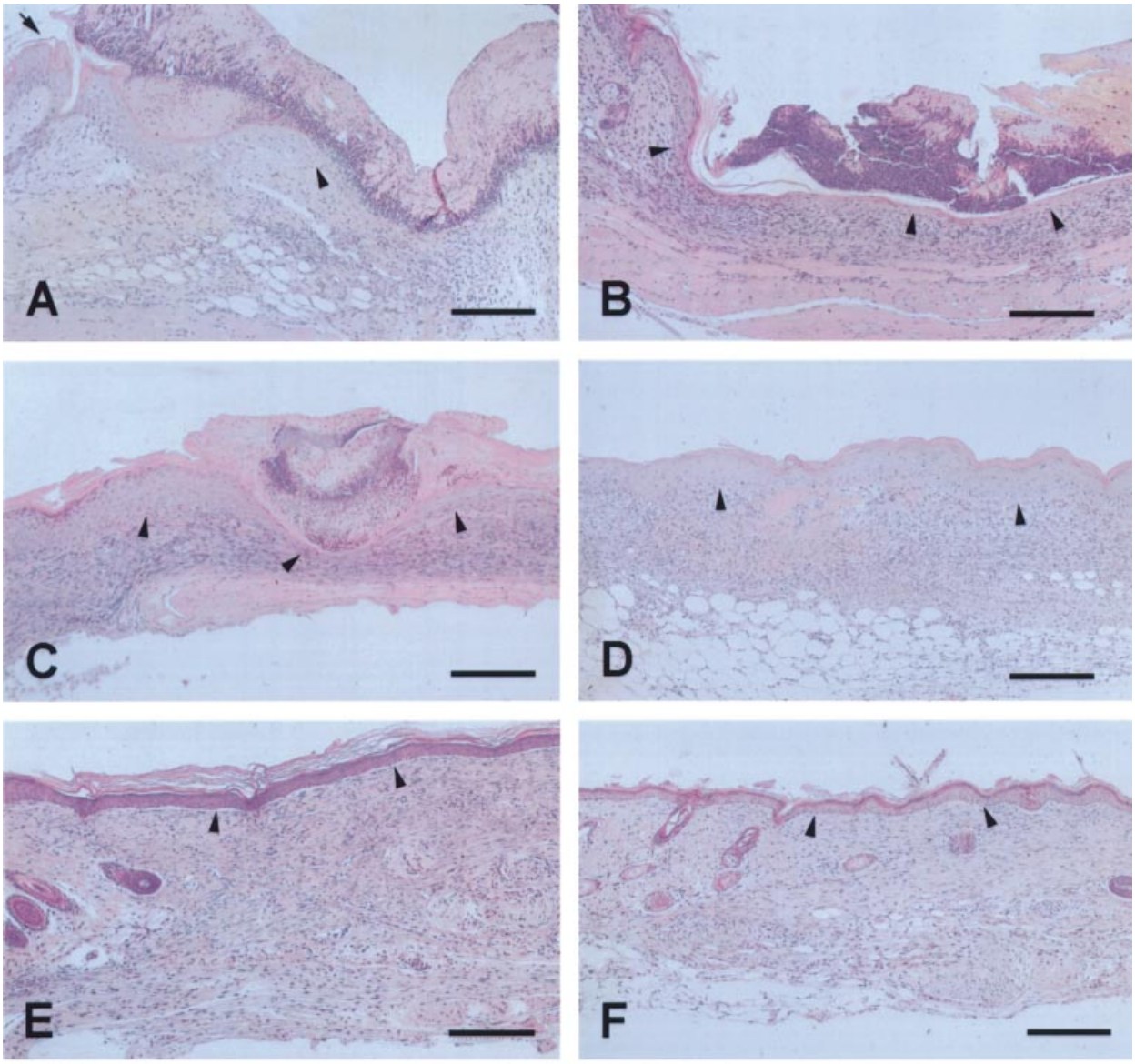
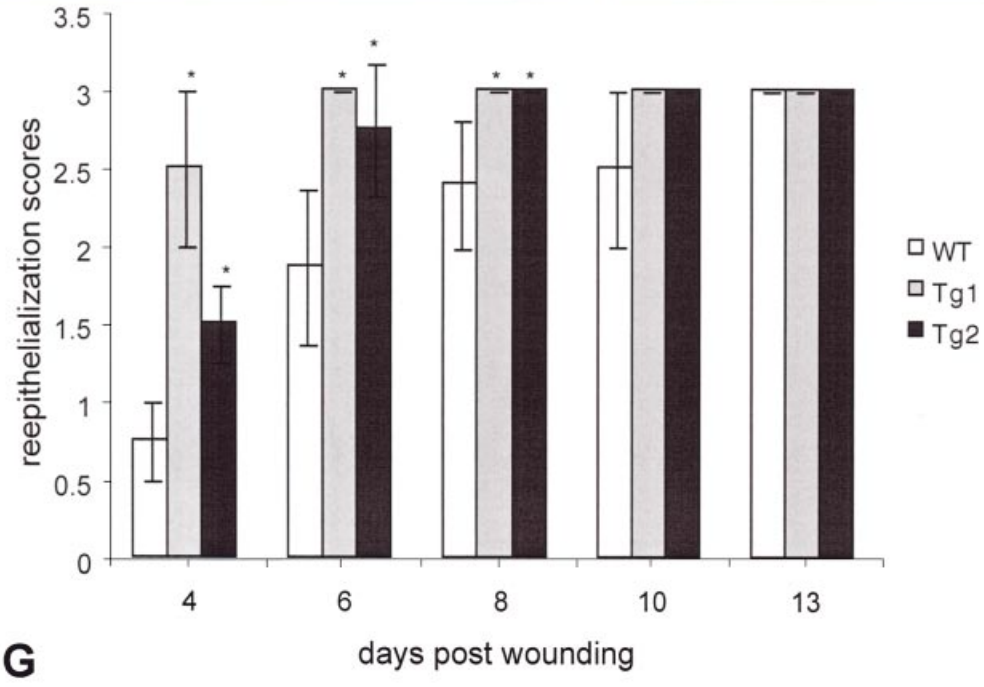


Figure 2. Scab rejection after wounding. At least five animals of each transgenic line, Tg1 and Tg2, as well as wild-type controls were wounded and examined daily for scab rejection. Note the early onset of scab rejection in both transgenic lines Tg1 and Tg2 compared to the wild-type animals.

Figure 3. Progression of wound healing. Animals of both transgenic lines Tg1 and Tg2 as well as control animals ($n > 5$ animals per group and time point) were wounded and wounds were removed and prepared for routine hematoxylin and eosin staining. (*A*) –(*F*) Hematoxylin and eosin stained sections from control animals (*A*, *C*, *E*) and transgenic animals of line Tg2 (*B*, *D*, *F*); at 4 d (*A*, *B*), 6 d (*C*, *D*), and 10 d (*E*, *F*) post wounding. Note the rapid progression of scab rejection, reepithelialization, granulation tissue formation, and tissue remodeling in transgenic animals *versus* controls. (*A*) *Arrow*, *arrowhead*: wound margin and migrating epithelial tongue, respectively; (*B–F*) *arrowheads*: newly formed epithelium. *Scale bars*: 200 μm. (*G*) Histogram showing reepithelialization scores. Hematoxylin and eosin stained sections were evaluated in a double blinded manner for the extent of reepithelialization, and scores ranging from I to III were allotted (I, marginal reepithelialization; II, moderate closure of wound; III, complete closure of wound) ($n = 10$ wounds per group and time point). Note the early closure of wounds in transgenic animals Tg1 and Tg2 compared to the wild-type animals.


A
B
C
D
E
F


3.5
3
2.5
2
1.5
1
0.5
0
reepithelialization scores
4
6
8
10
13
days post wounding
WT
Tg1
Tg2
G

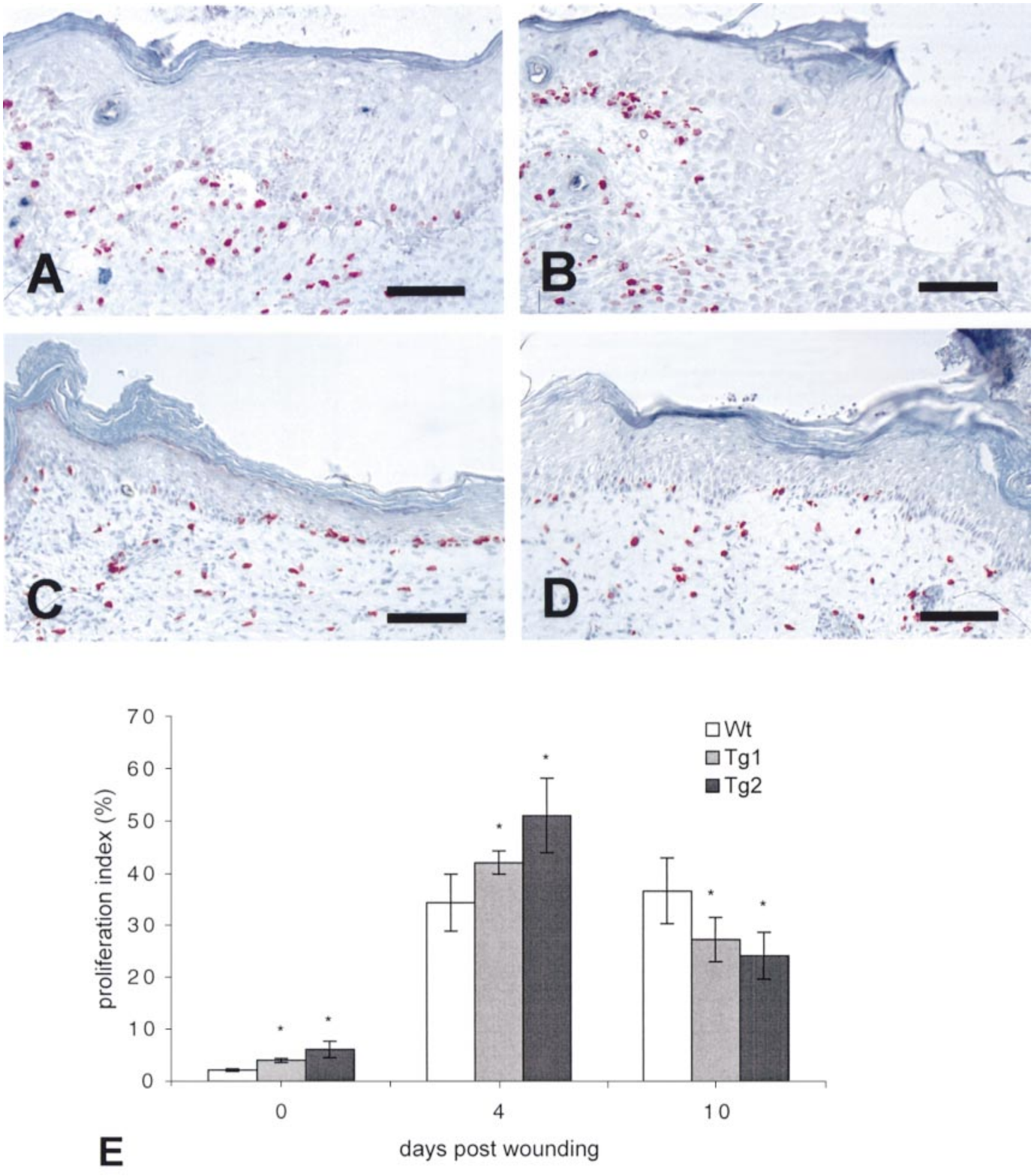


Figure 4. Mitotic indices during wound healing. (*A*)–(*D*) Mice were injected intraperitoneally with BrdUrd and sacrificed after a labeling period of 2 h ($n = 10$ wounds per experimental group and time point). Skin sections were stained for detection of BrdUrd-labeled S-phase nuclei. (*A*), (*C*) Control animals; (*B*), (*D*) transgenic animals at 4 and 10 d post wounding, respectively. Four days after wounding, transgenic animals (*B*) exhibit more labeled nuclei in the basal layer of the epidermis than the wild-type animals (*A*). By 10 d post wounding, however, these relations are reversed (*C*, *D*). *Scale bars*: 200 μm. (*E*) Histogram showing numbers of S-phase nuclei at the wound margin. BrdUrd-labeled nuclei were counted in the thickened wound margins and related to 100 total basal cells ($n = 10$ wounds per group and time point). Significantly higher numbers of proliferating keratinocytes in transgenics of lines Tg1 and Tg2 compared to the wild-type animals in unwounded skin as well as at 4 d post wounding can be seen. Ten days after wounding, controls exhibit significantly higher mitotic indices compared to the transgenic animals (*$p < 0.05$).

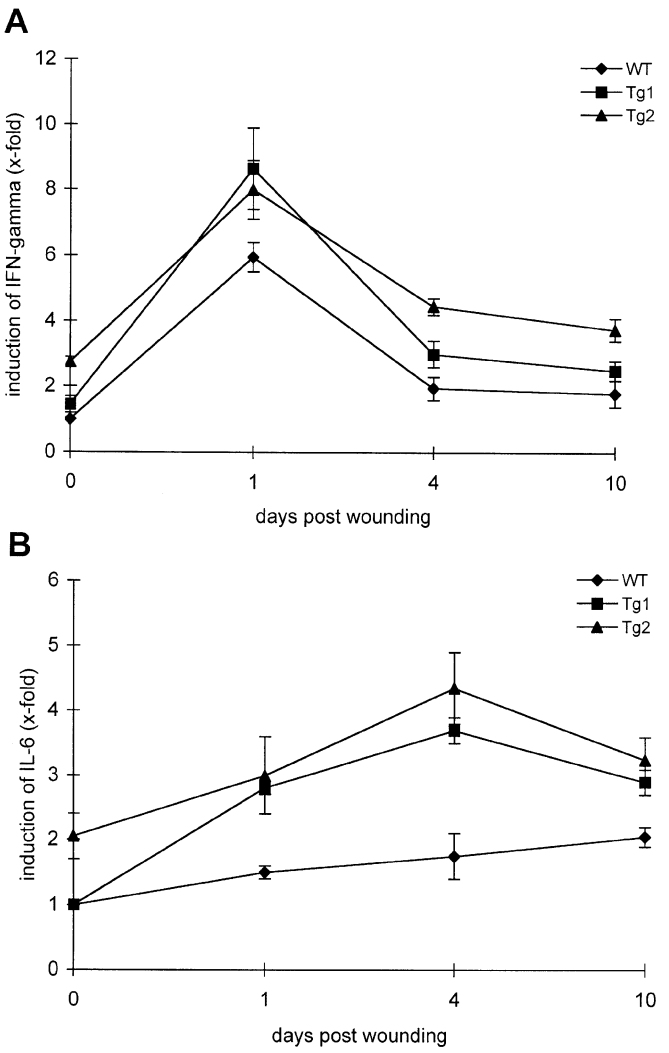


Figure 5. Temporal expression of IFN-γ and IL-6 transcripts during the repair process as determined by RNase protection assay. (*A*) IFN-γ; (*B*) IL-6. Mice of both transgenic lines as well as littermate controls were wounded and total RNA was prepared (four wounds were pooled per experimental time point). For one representative experiment, the relative expression levels of IFN-γ and IL-6 mRNA are shown as assessed by using a phosphoimager (Molecular Dynamics).

Gallucci *et al*, 2000), and IFN-γ were also upregulated in GM-CSF transgenic mice. We anticipate that this transgenic mouse model will be useful in studying benefits, perils, and mechanisms of GM-CSF in normal and impaired wound healing.

MATERIALS AND METHODS

Mice Transgenic mouse lines Tg1 and Tg2 overexpressing GM-CSF under the control of the bovine keratin 5 promoter have been described previously (Breuhahn *et al*, 2000; Mann *et al*, 2001). Transgenic mice were maintained as heterozygotes on an inbred FVB/N background. Wildtype siblings were used as experimental controls. All mice were bred and housed in the animal facility of the Johannes Gutenberg University in Mainz.

TPA application and wounding protocol Age and sex matched mice were shaved on the mid-dorsum 1 d prior to treatment. 2.5 μg TPA, dissolved in 200 μl acetone, were applied on the back. Animals were sacrificed at different time points after the treatment and dorsal skin was collected for cytokine analysis.

Animals aged 8–10 wk were anesthetized by avertin. The backs of the animals were shaved and swabbed with 70% ethanol. One 5 mm diameter full-thickness excisional wound per animal was inflicted on the mid-dorsum, using a biopsy punch. The wounds were left unsutured and without dressing. Subsequently, animals were housed individually. Four to five animals were analyzed for each time point and experiments were repeated twice.

Wound analysis The wounds were observed once every day for infection and for the rejection of scab. Animals were euthanized at 4, 8, 6, 10, 13, and 16 d after wounding. The wound area was cleaned carefully with 70% ethanol and the entire wound, including 4–5 mm marginal unwounded skin, was carefully excised. The wound was divided in two halves by a median section. One half was fixed in neutral buffered 4% formalin and processed for paraffin embedding. The other half was snap-frozen by overlaying with tissue-embedding medium (Jung, Leica Instruments, Nussloch, Germany) and immersing it immediately in liquid nitrogen. Tissues were stored at –80°C until analysis.

In addition, normal skin and wounded skin at 1, 4, 7, and 10 d post wounding were sampled, frozen in liquid nitrogen, and stored at –80°C for molecular analysis. At least five animals for each experimental group were taken per time point and the experiments were repeated twice.

Histology and immunohistology Sections were mounted on slides and stained routinely with hematoxylin and eosin. Evaluation of the sections was done in a double blind manner by two investigators, including a senior pathologist (PS). The wounds were evaluated for the extent of reepithelialization. Depending upon the area covered by keratinocytes, scores ranging from I to III (I, weak; II, moderate; III, complete reepithelialization of the wound) were allotted to each sample. Also, granulation tissue formation, architecture, cellularity, and inflammation were evaluated.

Cryosections were cut at 5 μm thickness and processed for immunostaining. Treatment of samples for immunohistologic procedures and antibodies used have been described previously (Breuhahn *et al*, 2000). In this study, an antibody directed to CD31 (PECAM-1, 1:100; Pharmingen, Germany) was used for the detection of microvessels in the wound bed. The secondary antibody was AP-conjugated and the signal detection was carried out using NBT/X-phosphate as a chromogenic substrate. The sections were photographed subsequently and the extent of microvessel formation during the repair process was determined.

Determination of mitotic indices Cell proliferation assay was carried out by BrdUrd (5-bromo-2′-deoxy-uridine) labeling experiments using the *In situ* Cell Proliferation Kit, AP, according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). The sections were counterstained with Mayer's hemalum solution (Merck, Darmstadt, Germany). The number of labeled nuclei in the basal layer of the epidermis at the wound margin was determined and related to 100 total basal cells. Values were obtained from at least five animals per experimental group and time point.

Cytokine determination Tissue extracts were prepared from the skin samples essentially as described previously (Breuhahn *et al*, 2000). Total amount of protein was determined using Bradford reagent (Biorad, Munich, Germany). Concentrations of GM-CSF were determined using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN).

Northern blot analysis Northern blot analysis was done essentially as described previously (Breuhahn *et al*, 2000). The hybridization probes were generated by polymerase chain reaction (PCR) as described previously (Breuhahn *et al*, 2000) with murine spleen cDNA serving as template and using the following primer pairs: TGF-β1: 5tgfb1 (5′-CAT GAA TTC CTG GCG TTA CCT TGG-3′; position 1396–1415) and 3tgfb1 (5′-GCT CGT GGA TCC ACT TCC AAC C-3′; position 1786–1807; GenBank accession no. NM 011577).

RNase protection assay The ribonuclease protection assays were carried out using Riboquant™ Multi-Probe RNase Protection assay system (Pharmingen, San Diego, CA). The system included an *In vitro* Transcription Kit, the RPA Kit, and the Multi-Probe Template Sets mCK-3, mCK-3b, and mAPO-2. Ribonuclease protection assays were carried out according to the manufacturer's instructions, using pooled total RNA from at least three animals per experimental group. Total RNA was isolated from the wound specimens using peqGOLD RNA Pure (Peqlab Biotechnologie, Erlangen, Germany).

The band intensities were quantified using the Image Quant System (Molecular Dynamics, Sunnyvale, CA). The band intensities of different

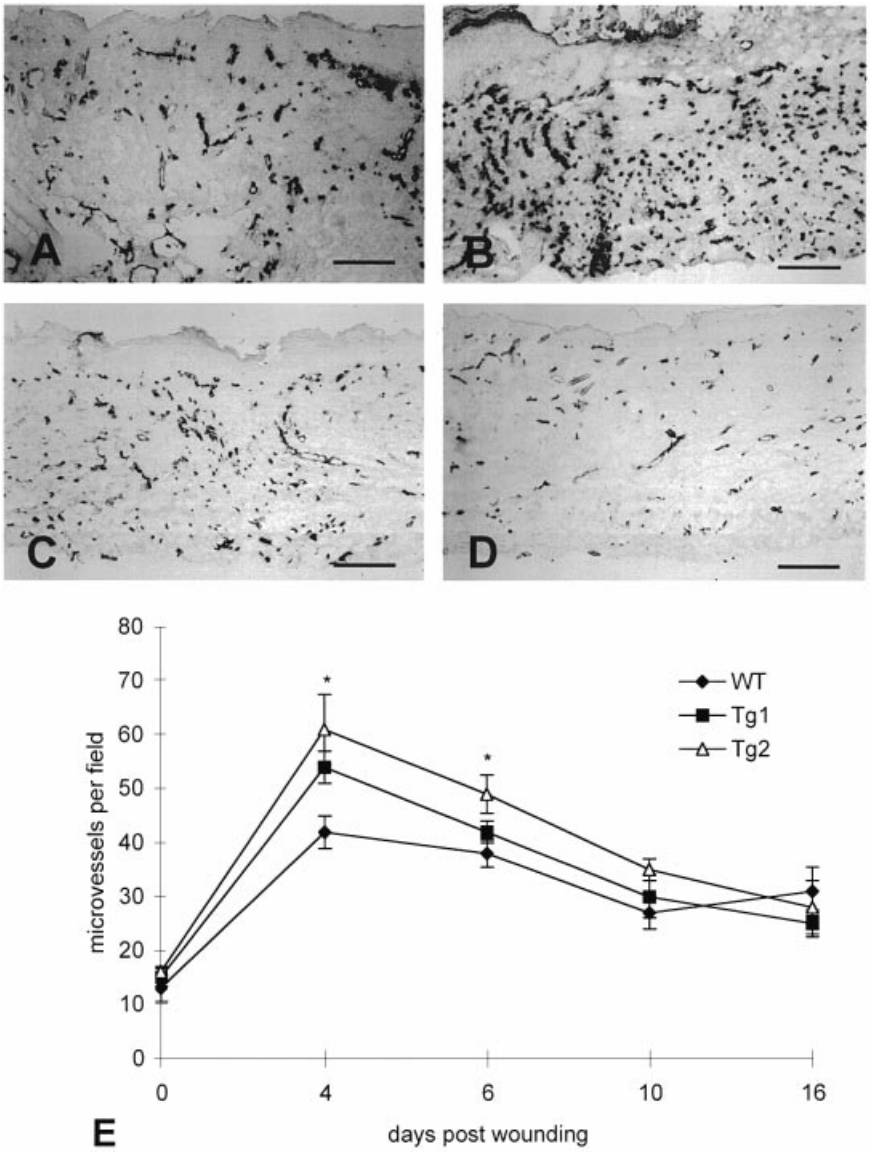


Figure 6. Extent of neovascularization during the repair process. (*A–D*) Skin sections were stained with a CD31-specific antibody for detection of blood vessels ($n > 5$ wounds per group and time point). Increased numbers of blood vessels are visible in transgenic animals compared to the wild-type animals, 4 d post wounding (*A*, *B*). At 16 d post wounding, however, these differences disappeared (*C*, WT; *D*, transgenics). *Scale bars*: 200 μm. (*E*) Graphical representation of numbers of newly formed blood vessels in the wound bed. Stained samples were photographed and the numbers of microvessels per field were counted. Significantly higher numbers of blood vessels are formed at days 4 and 6 after wounding in the transgenic lines Tg1 and Tg2 compared to the wild-type controls (*$p < 0.05$).

mRNA species were related to the band intensities of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or the ribosomal protein L32.

Statistics Data are shown as means ± SD. Statistical significance in the differences between two groups was analyzed using Student's *t* test and values of $p < 0.05$ were considered significant.

RESULTS

GM-CSF is upregulated following treatment with TPA or wounding On the RNA level, it had been shown previously that GM-CSF is induced in proliferating keratinocytes and that GM-CSF is an early response factor following treatment with tumor promoting agents (Robertson *et al*, 1994; Vasunia *et al*, 1994; Pastore *et al*, 1997; Park *et al*, 2001). The main cell type responsible for the GM-CSF production in these studies has been shown to be keratinocytes. For analysis of GM-CSF induction kinetics on the protein level in skin we determined by ELISA tissue concentrations for this cytokine at 3, 6, 8, 12, 18, 24, 48, and 72 h post TPA application in wild-type mice. Highest levels of GM-CSF were measured as soon as 6 h after the treatment and declined therafter (**Fig 1*A***). GM-CSF levels in skin at 1, 7, and 10 d post wounding were elevated in transgenic, GM-CSF-overexpressing mice of lines Tg1 and Tg2 in comparison to controls (**Fig 1*B***). The wild-type controls showed undetectable GM-CSF levels in normal skin and moderate GM-CSF levels following wounding.

GM-CSF transgenics exhibit accelerated scab rejection Nine to ten animals of each line as well as littermate controls were wounded and inspected daily for the general appearance of the wound, presence of infection, and scab rejection. Within 24–48 h after wounding, most of the wounds in the animals of both transgenic lines Tg1 and Tg2 had a dry appearance and were covered with a scab, whereas the majority of wounds from the wild-type animals still had a glistening moist appearance and were covered with a thin scab. Significant differences were observed in the rate of scab rejection between transgenic lines and control animals. First scabs were rejected in lines Tg1 and Tg2 already 6 d post wounding (**Fig 2**). Seven days post wounding 25% and 60% of scabs were rejected in lines Tg1 and Tg2 compared to only 10% scab rejection in the animals of the control group. On day 9 post wounding, 90% of the scabs in both the transgenic lines had fallen off, compared to 60% scab rejection in the wild-type animals.GM-CSF overexpression enhances reepithelialization and tissue remodeling Sections through the center of the wounds excised at 4, 6, 8, 10, 13, and 16 d after wounding were stained with hematoxylin and eosin and the extent and rate of reepithelialization was evaluated. Differences were observed in the rate of

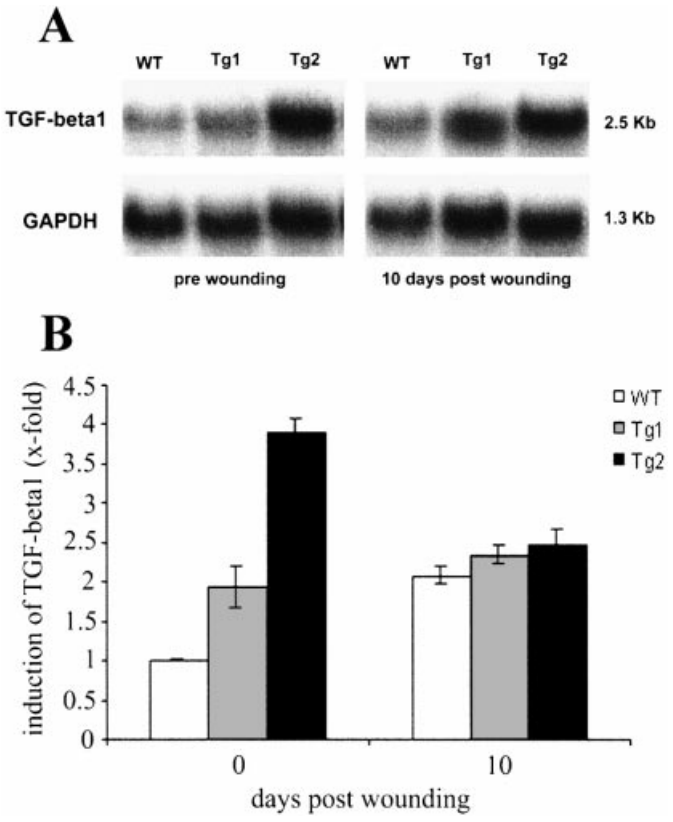


Figure 7. Expression of TGF-β1 mRNA. Northern blot analysis was performed in order to investigate the temporal regulation of TGF-β1 transcripts during the repair process. Total RNA from four to five wounds were pooled per experimental time point. Increased basal levels of TGF-β1 mRNA were detected in Tg1 and Tg2 (*A*). Quantitative evaluation of TGF-β1 expression in relation to GAPDH expression (*B*). Note the higher level of TGF-β1 expression in unwounded skin of transgenics in comparison to controls.

reepithelialization between the transgenic animals and the control wild-type animals as exemplified in **Fig 3**(***A***)–(***F***). On a quantitative basis, both transgenic lines Tg1 and Tg2 reached significantly higher reepithelialization scores in comparison to controls (**Fig 3*G***). Whereas reepithelialization was complete in mice of lines Tg1 and Tg2 at days 6 and 8 post wounding, respectively, complete closure of the wounded area was reached in the wild-type control group only by 13 d post wounding (**Fig 3*G***).

Besides reepithelialization, abundant granulation tissue with a very high cellularity was present in the wound bed in transgenics on day 4 post wounding (**Fig 3*B***). By contrast, comparable abundance and cellularity of granulation tissue could be observed in the wild-type mice only at 6 d post wounding (**Fig 3*C***). Similarly, the decrease in dermal cellularity was delayed in wild-type mice *versus* transgenics resulting in a significantly higher cellularity at 10 d post wounding in the control group compared to the transgenic animals (**Fig 3*E*, *F***). This enhanced tissue remodeling at the later stages of wound repair in the transgenic animals was also reflected by the presence of hair follicles in the wound bed in the transgenic animals, which were absent at this stage in controls (**Fig 3*E*, *F***).

Mitotic indices are augmented in GM-CSF transgenics Mice of transgenic lines Tg1 and Tg2 exhibit increased mitotic indices in the interfollicular epidermis, by a factor of approximately 2–3 compared to control mice (Breuhahn *et al*, 2000). Proliferation rates at the wound edge were measured by BrdUrd labeling at 4 and 10 d after wounding as exemplified in **Fig 4**(***A***)–(***D***). Both transgenic lines exhibit an early proliferative burst, with maximum proliferation rates being achieved 4 d post wounding. Significantly higher numbers of proliferating basal keratinocytes, 42% in Tg1 and 51% in Tg2 compared to 34% in the wild-type controls, were measured on day 4 after wounding (**Fig 4*A*, *B*, *E***). High proliferation rates in the wild-type controls, albeit on a lower level than the maximum proliferation rates in transgenics, were maintained longer in comparison to transgenics. Therefore, on day 10 after wounding, wild-type mice exhibited higher numbers of proliferating keratinocytes compared to the transgenic animals (**Fig 4*C*, *D*, *E***). Thus, the strong and early burst in keratinocyte proliferation is followed by an earlier downregulation of proliferation in transgenics *versus* controls.

Proinflammatory cytokines are induced in GM-CSF transgenics Expression of mRNAs of different cytokines relevant to cutaneous inflammation and fibrosis was measured using a multitemplate set RNase protection assay including TNF-α, TNF-β, IL-6, IFN-γ, IFN-β, TGF-β1, TGF-β2, TGF-β3, MIF, LT-β, as well as housekeeping gene transcripts L32 and GAPDH. Significant differences between transgenics and controls in the induction of mRNA for IL-6 and IFN-γ were detected during the course of the wound repair process. The inductions of IFN-γ (**Fig 5*A***) and IL-6 (**Fig 5*B***) were several-fold higher in transgenic animals compared to the wild-type littermates. No significant differences in the temporal regulation of other cytokines like M-CSF, IL-3, and TNF-α were observed (data not shown).

GM-CSF transgenics exhibit enhanced neovascularization after wounding Sections were stained with an antibody directed against CD31 and the extent of microvessel formation during the repair process was determined (**Fig 6*A*–*E***). CD31-positive microvessels were counted and expressed as numbers per field. The number of newly formed capillaries was significantly higher in both transgenic lines 4 d post wounding compared to the control group (**Fig 6*A*, *B***). Transgenic mice of lines Tg1 and Tg2 exhibited 54 and 61 microvessels per vision field, respectively, compared to 42 microvessels per field in the wild-type mice (**Fig 6*E***). After day 4 post wounding the numbers of microvessels gradually decreased in both transgenics and the nontransgenic controls. A faster decline in transgenics, however, leads to an approximation of microvessel density between transgenic animals and control animals at day 16 post wounding (**Fig 6*C*–*E***).

TGF-β1 expression is differentially regulated during the repair process As TGF-β1 has been shown to play an important role in the process of wound healing, levels of TGF-β1 mRNA were determined by RNase protection assay (data not shown) and northern blot analysis (**Fig 7*A***). The band intensities were equalized by comparison to the band intensities for GAPDH and a time lapse profile was conveyed (**Fig 7*B***). Transgenic mice of lines Tg1 and Tg2 exhibited two to four times higher levels of TGF-β1 mRNA compared to control mice in unwounded skin. During wound healing, mRNA levels of this isoform were also elevated at least 2-fold in transgenics as well as in controls as exemplified by the 10 d post wounding time point (**Fig 7*B***).

DISCUSSION

Poorly healing and chronic wounds constitute a major challenge to clinical care as these ailments greatly contribute to the morbidity of patients from diverse disciplines including dermatology, oncology, hematology, and surgery. The process of wound healing is a highly organized event resulting in the restoration of an intact barrier function of the skin. It involves interactions of many different cell types, matrix components, and biochemical factors. GM-CSF is a possible candidate for a central growth factor because it is synthesized by a number of cellular components of the repair process, e.g., keratinocytes, endothelial cells, fibroblasts, dendritic cells, and macrophages (Dedhar *et al*, 1988; Bussolino *et al*, 1989, 1991; Caux *et al*, 1992; Braunstein *et al*, 1994; Breuhahn *et al*, 2000). In normal skin GM-CSF is not synthesized by keratinocytes in sizeable amounts. Upregulation of GM-CSF has been shown to be an early event after injury or treatment with a tumor promoting

agent, however (Robertson *et al*, 1994; Vasunia *et al*, 1994; Pastore *et al*, 1997). Consistent with these observations, we found a strong upregulation of GM-CSF at the protein level in murine skin shortly after irritation. At 4 h post activation, the earliest timepoint analyzed, significant tissue levels for GM-CSF were measured. The peak of GM-CSF tissue concentration was found around 6 h post irritation followed by a gradual decrease over several days. Thus, GM-CSF has to be considered an early response factor in skin regeneration.

So far, the effects of GM-CSF in the process of wound healing have been discussed controversially. On the one hand it has been shown to improve healing of chronic wounds and ulcers of different etiology (Kaplan *et al*, 1992; Kaplan, 1993; El Saghir *et al*, 1997; Jaschke *et al*, 1999). On the other hand, it has been reported to have no effect on the healing process in healthy individuals (Ure *et al*, 1998). In most cases, GM-CSF was applied either topically or by perilesional injections. In this study, we have analyzed the process of cutaneous wound healing in a transgenic mouse model with constitutive overexpression of GM-CSF by keratinocytes in unwounded skin (Breuhahn *et al*, 2000). We were able to show that, at all stages of wound repair, GM-CSF tissue levels were several-fold higher in transgenic animals *versus* controls. This model was used to examine the effects of a continuous excess of keratinocyte-derived GM-CSF on wound repair.

We started out to analyze the process of normal cutaneous wound healing in these animals. Healing of full-thickness excisional wounds as measured by the time required for scab rejection and for the complete reepithelialization of the wounds was enhanced in transgenic mice in comparison to the littermate controls. We propose that there are several distinct mechanisms by which GM-CSF accelerates wound healing in our transgenic mouse model.

(i) GM-CSF has been shown to possess positive effects on keratinocyte growth *in vitro* and *in vivo* (Hancock *et al*, 1988; Braunstein *et al*, 1994; Breuhahn *et al*, 2000). It is known to stimulate proliferation and migration of human keratinocytes and endothelial cells (Bussolino *et al*, 1989; Kawada *et al*, 1997). In accordance with these findings, we observed a stronger induction of keratinocyte proliferation early after wounding in our transgenic mice compared to control mice. This hyperproliferation in conjunction with enhanced migratory capabilities of keratinocytes most probably accounts for the rapid influx of keratinocytes into the wounded site in transgenics resulting in accelerated reepithelialization. We attribute this hyperproliferative response in transgenics mainly to post wounding stimuli. The activation state of keratinocytes in the transgenic animals in the unwounded state is low despite the observed hyperproliferation as determined by the absence of stress marker keratin 6/16 (Breuhahn *et al*, 2000).

(ii) Besides reepithelialization, the infiltration of various cell types into the wound area and the subsequent formation of a thorough granulation tissue is a prerequisite for successful wound repair. Transient expression of GM-CSF using a replication-deficient adenoviral vector resulted in inflammation and massive infiltration of the site as well as epidermal hyperplasia (Rajagopalan *et al*, 1995; Xing *et al*, 1997a). GM-CSF has been shown to be chemotactic for a number of cells like Langerhans cells, macrophages, neutrophils, fibroblasts, and endothelial cells (Ruef and Coleman, 1990; Bussolino *et al*, 1991; Jones, 1993; Braunstein *et al*, 1994; Xing *et al*, 1997b). Thus, more abundant granulation tissue formation with higher cellularity was present earlier after wounding in the transgenic animals than in littermate controls.

(iii) Neovascularization of the wound area is pivotal for the supply of components essential for tissue repair as well as for the disposal of debris. GM-CSF has been shown to induce proliferation and migration of endothelial cells (Bussolino *et al*, 1991). Consistent with these findings, we observed both accelerated and increased neovascularization of the wound bed in transgenic animals *versus* controls.

Although some of these actions such as autocrine stimulation of keratinocyte proliferation can be directly brought about by GM-CSF itself (Breuhahn *et al*, 2000), other effects are most probably invoked by the GM-CSF-mediated release of other cytokines and growth factors as demonstrated in our study for IFN-γ, IL-6, and TGF-β1. IFN-γ is a central, proinflammatory cytokine that has activating and chemoattractant activities on many cell types required for granulation tissue formation (Valente *et al*, 1998; Gasperini *et al*, 1999; Li *et al*, 2000). Thus, the higher level of IFN-γ expression in transgenics *versus* controls may account for the accelerated and more intense formation of granulation tissue in these animals. Likewise, the necessity of IL-6 and activation of the STAT-3 signaling pathway for wound healing is well established (Sano *et al*, 1999; Gallucci *et al*, 2000). It has been shown previously that IL-6 expression is upregulated following injury (Pajulo *et al*, 1999). It is produced mainly by keratinocytes, but also by macrophages, Langerhans cells, and fibroblasts (Paquet and Pierard, 1996).

An important aspect of this study is the induction of TGF-β1 by GM-CSF. It had been shown previously that GM-CSF overexpression in lungs of rats leads to a marked increase in TGF-β1 protein levels in bronchioalveolar lavage fluids (Xing *et al*, 1996; 1997b). TGF-β1 has been well documented as a potent growth inhibitor for epithelial cells *in vitro* and *in vivo* (Glick *et al*, 1993; Wang *et al*, 1997; Wang D *et al*, 1999; Wang XJ *et al*, 1999). In contrast, numerous animal studies have shown that exogenously applied TGF-β1 enhances epidermal wound healing (Mustoe *et al*, 1991; Schmid *et al*, 1993). Initiation of a number of events essential for wound healing such as production of cytokines and inflammatory mediators, chemoattraction of monocytes and leukocytes, regulation of fibroblast function, induction of angiogenesis, and modulation of synthesis of proteases and extracellular matrix have been attributed to TGF-β1 (Moses *et al*, 1990; Quaglino *et al*, 1990, 1991; Salo *et al*, 1991; Moses, 1992; Border and Noble, 1994).

It is notable that the acceleration of reepithelialization and intensification of granulation tissue formation does not alter the subsequent succession of events at later stages of wound healing, e.g., dissolution of granulation tissue, remodeling, and wound contraction. This is also reflected by the downregulation of TGF-β1 expression in transgenics *versus* controls at day 10 post wounding. The more advanced stage of wound repair in these animals corresponds to declined TGF-β1 expression levels (Frank *et al*, 1996). The effect of a continuous surplus of keratinocyte-derived GM-CSF appears to be beneficial in normal wound healing. The discrepancy in the one report that found no effect of GM-CSF on normal wound healing may be explained by the singular application of this cytokine in the underlying study (Ure *et al*, 1998). Our animal model is now being used to resolve this discrepancy.

This work was funded by the DFG in project B1 of program grant SFB432 and the Boehringer Ingelheim Foundation. We are indebted to Prof. Dr. Galle for constant support and encouragement and to Katharina Petmecky for excellent technical assistance.

REFERENCES

Baker EA, Leaper DJ: Proteinases, their inhibitors, and cytokine profiles in acute wound fluid. *Wound Repair Regen* 8:392–398, 2000

Border WA, Noble NA: Transforming growth factor beta in tissue fibrosis. *N Engl J Med* 331:1286–1292, 1994

Bratton DL, Hamid Q, Boguniewicz M, Doherty DE, Kailey JM, Leung DY: Granulocyte macrophage colony-stimulating factor contributes to enhanced monocyte survival in chronic atopic dermatitis. *J Clin Invest* 95:211–218, 1995

Braunstein S, Kaplan G, Gottlieb AB, *et al*: GM-CSF activates regenerative epidermal growth and stimulates keratinocyte proliferation in human skin *in vivo*. *J Invest Dermatol* 103:601–604, 1994

Breuhahn K, Mann A, Müller K, Wilhelmi A, Schirmacher P, Enk A, Blessing M: Overexpression of GM-CSF in the epidermis of transgenic mice induces both keratinocyte proliferation and apoptosis. *Cell Growth Differentiation* 11:111–121, 2000

Bussolino F, Wang JM, Defilippi P, *et al*: Granulocyte- and granulocyte-macrophage-

colony stimulating factors induce human endothelial cells to migrate and proliferate. *Nature* 337:471–473, 1989

Bussolino F, Ziche M, Wang JM, *et al*: *In vitro* and *in vivo* activation of endothelial cells by colony-stimulating factors. *J Clin Invest* 87:986–995, 1991

Caux C, Dezutter-Dambuyant C, Schmitt D, Banchereau J: GM-CSF and TNF-alpha cooperate in the generation of dendritic Langerhans cells. *Nature* 360:258–261, 1992

Caux C, Vanbervliet B, Massacrier C, et al: CD34+ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to GM-CSF+TNF alpha. *J Exp Med* 184:695–706, 1996

Clark RA: Cutaneous tissue repair: basic biologic considerations. I. *J Am Acad Dermatol* 13:701–725, 1985

Da Costa RM, Ribeiro Jesus FM, Aniceto C, Mendes M: Randomized, double-blind, placebo-controlled, dose-ranging study of granulocyte-macrophage colony stimulating factor in patients with chronic venous leg ulcers. *Wound Repair Regen* 7:17–25, 1999

Dedhar S, Gaboury L, Galloway P, Eaves C: Human granulocyte-macrophage colony-stimulating factor is a growth factor active on a variety of cell types of nonhemopoietic origin. *Proc Natl Acad Sci USA* 85:9253–9257, 1988

El Saghir NS, Bizri AR, Shabb NS, Husami TW, Salem Z, Shamseddine AI: Pressure ulcer accelerated healing with local injections of granulocyte macrophage-colony stimulating factor. *J Infect* 35:179–182, 1997

Frank S, Madlener M, Werner S: Transforming growth factors beta1, beta2, and beta3 and their receptors are differentially regulated during normal and impaired wound healing. *J Biol Chem* 271:10188–10193, 1996

Gaches F, Blanc AS, Couret B, Arlet-Suau E: Digital necroses and Sharp's syndrome: the success of topical application of granulocyte/macrophage-colony stimulating factor in promoting healing after amputation of three toes. *Br J Dermatol* 138:550–551, 1998

Gallucci RM, Simeonova PP, Matheson JM, Kommineni C, Guriel JL, Sugawara T, Luster MI: Impaired cutaneous wound healing in interleukin-6-deficient and immunosuppressed mice. *Faseb* 14:2525–2531, 2000

Gasperini S, Marchi M, Calzetti F, et al: Gene expression and production of the monokine induced by IFN-gamma (MIG), IFN-inducible T cell alpha chemoattractant (I-TAC), and IFN-gamma-inducible protein-10 (IP-10) chemokines by human neutrophils. *J Immunol* 162:4928–4937, 1999

Glick AB, Kulkarni AB, Tennenbaum T, et al: Loss of expression of transforming growth factor beta in skin and skin tumors is associated with hyperproliferation and a high risk for malignant conversion. *Proc Natl Acad Sci USA* 90:6076–6080, 1993

Grose R, Sabine W: *Wound Healing Studies in Transgenic and Knockout Mice*. Humana Press, 2001, in press

Hancock GE, Kaplan G, Cohn ZA: Keratinocyte growth regulation by the products of immune cells. *J Exp Med* 168:1395–1402, 1988

Jaschke E, Zabernigg A, Gattringer C: Recombinant human granulocyte-macrophage colony-stimulating factor applied locally in low doses enhances healing and prevents recurrence of chronic venous ulcers. *Int J Dermatol* 38:380–386, 1999

Jones TC: The effects of rhGM-CSF on macrophage function. *Eur J Cancer* 29A:S10–S13, 1993

Jyung RW, Mustoe JA, Busby WH, Clemmons DR: Increased wound-breaking strength induced by insulin-like growth factor I in combination with insulin-like growth factor binding protein-1. *Surgery* 115:233–239, 1994a

Jyung RW, Wu L, Pierce GF, Mustoe TA: Granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor: differential action on incisional wound healing. *Surgery* 115:325–334, 1994b

Kaplan G: Recent advances in cytokine therapy in leprosy. *J Infect Dis* 167:S18–S22, 1993

Kaplan G, Walsh G, Guido LS, *et al:* Novel responses of human skin to intradermal recombinant granulocyte/macrophage-colony-stimulating factor: Langerhans cell recruitment, keratinocyte growth, and enhanced wound healing. *J Exp Med* 175:1717–1728, 1992

Kawada A, Hiruma M, Noguchi H, Ishibashi A, Motoyoshi K, Kawada I: Granulocyte and macrophage colony-stimulating factors stimulate proliferation of human keratinocytes. *Arch Dermatol Res* 289:600–602, 1997

Li Q, Normolle DP, Sayre DM, *et al*: Immunological effects of BCG as an adjuvant in autologous tumor vaccines. *Clin Immunol* 94:64–72, 2000

Mann A, Breuhahn K, Schirmacher P, *et al*: Up- and downregulation of granulocyte-macrophage colony-stimulating factor activity in murine skin increase susceptibility to skin carcinogenesis by independent mechanisms. *Cancer Res* 61:2311–2319, 2001

Martin P: Wound healing – aiming for perfect skin regeneration. *Sci* 276:75–81, 1997

Moses HL: TGF-beta regulation of epithelial cell proliferation. *Mol Reprod Dev* 32:179–184, 1992

Moses HL, Yang EY, Pietenpol JA: TGF-beta stimulation and inhibition of cell proliferation: new mechanistic insights. *Cell* 63:245–247, 1990

Mustoe TA, Pierce GF, Morishima C, Deuel TF: Growth factor-induced acceleration of tissue repair through direct and inductive activities in a rabbit dermal ulcer model. *J Clin Invest* 87:694–703, 1991

Pajulo OT, Pulkki KJ, Alanen MS, Reunanen MS, Lertola KK, Mattila-Vuori AI, Viljanto JA: Correlation between interleukin-6 and matrix metalloproteinase-9 in early wound healing in children. *Wound Repair Regen* 7:453–457, 1999

Paquet P, Pierard GE: Interleukin-6 and the skin. *Int Arch Allergy Immunol* 109:308–317, 1996

Park KC, Kim DS, Kim HJ, et al: Growth related secretion and production of GM-CSF by epithelial cell line. *J Dermatol Sci* 25:53–58, 2001

Pastore S, Fanales-Belasio E, Albanesi C, Chinni LM, Giannetti A, Girolomoni G: Granulocyte macrophage colony-stimulating factor is overproduced by keratinocytes in atopic dermatitis. Implications for sustained dendritic cell activation in the skin. *J Clin Invest* 99:3009–3017, 1997

Quaglino D Jr, Nanney LB, Kennedy R, Davidson JM: Transforming growth factor-beta stimulates wound healing and modulates extracellular matrix gene expression in pig skin. I. Excisional wound model. *Laboratory Invest* 63:307–319, 1990

Quaglino D Jr, Nanney LB, Ditesheim JA, Davidson JM: Transforming growth factor-beta stimulates wound healing and modulates extracellular matrix gene expression in pig skin: incisional wound model. *J Invest Dermatol* 97:34–42, 1991

Rajagopalan LE, Burkholder JK, Turner J, Culp J, Yang NS, Malter JS: Granulocyte-macrophage colony-stimulating factor mRNA stabilization enhances transgenic expression in normal cells and tissues. *Blood* 86:2551–25581995

Rees RS, Robson MC, Smiell JM, Perry BH: Becaplermin gel in the treatment of pressure ulcers: a phase II randomized, double-blind, placebo-controlled study. *Wound Repair Regen* 7:141–147, 1999

Robertson FM, Bijur GN, Oberyszyn AS, Pellegrini AE, Boros LG, Sabourin CL, Oberyszyn TM: Granulocyte-macrophage colony stimulating factor gene expression and function during tumor promotion. *Carcinogenesis* 15:1017–1029, 1994

Ruef C, Coleman DL: Granulocyte-macrophage colony-stimulating factor: pleiotropic cytokine with potential clinical usefulness. *Rev Infect Dis* 12:41–62, 1990

Salo T, Lyons JG, Rahemtulla F, Birkedal-Hansen H, Larjava H: Transforming growth factor-beta 1 up-regulates type IV collagenase expression in cultured human keratinocytes. *J Biol Chem* 266:11436–11441, 1991

Sano S, Itami S, Takeda K, et al: Keratinocyte-specific ablation of Stat3 exhibits impaired skin remodeling, but does not affect skin morphogenesis. *Embo J* 18:4657–4668, 1999

Schmid P, Cox D, Bilbe G, et al: TGF-beta s and TGF-beta type II receptor in human epidermis: differential expression in acute and chronic skin wounds. *J Pathol* 171:191–197, 1993

Stagno F, Guglielmo P, Consoli U, Fiumara P, Russo M, Giustolisi R: Successful healing of hydroxyurea-related leg ulcers with topical granulocyte-macrophage colony-stimulating factor. *Blood* 94:1479–14801999

Ure I, Partsch B, Wolff K, Petzelbauer P: Granulocyte/macrophage colony-stimulating factor increases wound-fluid interleukin 8 in normal subjects but does not accelerate wound healing. *Br J Dermatol* 138:277–282, 1998

Valente AJ, Xie JF, Abramova MA, Wenzel UO, Abboud HE, Graves DT: A complex element regulates IFN-gamma-stimulated monocyte chemoattractant protein-1 gene transcription. *J Immunol* 161:3719–37281998

Vasunia KB, Miller ML, Puga A, Baxter CS: Granulocyte-macrophage colony-stimulating factor (GM-CSF) is expressed in mouse skin in response to tumor-promoting agents and modulates dermal inflammation and epidermal dark cell numbers. *Carcinogenesis* 15:653–660, 1994

Voskaridou E, Kyrtsonis MC, Loutradi-Anagnostou A: Healing of chronic leg ulcers in the hemoglobinopathies with perilesional injections of granulocyte-macrophage colony-stimulating factor. *Blood* 93:3568–3569, 1999

Wang D, Sun L, Zborowska E, Willson JK, Gong J, Verraraghavan J, Brattain MG: Control of type II transforming growth factor-beta receptor expression by integrin ligation [In Process Citation]. *J Biol Chem* 274:12840–12847, 1999

Wang X, Zinkel S, Polonsky K, Fuchs E: Transgenic studies with a keratin promoter-driven growth hormone transgene: prospects for gene therapy. *Proc Natl Acad Sci USA* 94:219–226, 1997.

Wang XJ, Liefer KM, Greenhalgh DA, Roop DR: 12-O-tetradecanoylphorbol-13-acetate promotion of transgenic mouse epidermis coexpressing transforming growth factor-alpha and v-fos: acceleration of autonomous papilloma formation and malignant conversion via c-Ha-ras activation. *Mol Carcinog* 26:305–311, 1999

Xing Z, Braciak T, Ohkawara Y, et al: Gene transfer for cytokine functional studies in the lung: the multifunctional role of GM-CSF in pulmonary inflammation. *J Leukoc Biol* 59:481–488, 1996

Xing Z, Gauldie J, Tremblay GM, Hewlett BR, Addison C: Intradermal transgenic expression of granulocyte-macrophage colony-stimulating factor induces neutrophilia, epidermal hyperplasia, Langerhans' cell/macrophage accumulation, and dermal fibrosis. *Laboratory Invest* 77:615–622, 1997a

Xing Z, Tremblay GM, Sime PJ, Gauldie J: Overexpression of granulocyte-macrophage colony-stimulating factor induces pulmonary granulation tissue formation and fibrosis by induction of transforming growth factor-beta 1 and myofibroblast accumulation. *Am J Pathol* 150:209–222, 1997b